Changes in the Concentrations of Hepatic Metabolites on Administration of Dihydroxyacetone or Glycerol to Starved Rats and their Relationship to the Control of Ketogenesis

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1. Glycerol and dihydroxyacetone, both antiketogenic and readily metabolized, but differing in their effects on the redox state of the hepatic NAD couples, were given to starved rats and the contents of metabolites were measured in freeze-clamped liver and in the blood. The object was to study the effects of changes in the redox state and of the availability of oxidizable substrates on the rate of ketone-body formation. 2. Intramuscular administration of dihydroxyacetone, glycerol or glucose to starved rats decreased the concentrations of acetoacetate and 3-hydroxybutyrate in the blood by 70–80% within 60 min., whereas there was no major change in the free fatty acid concentration. 3. Dihydroxyacetone, but not glucose or glycerol, caused an immediate and sustained twofold increase in the blood lactate concentration. 4. Dihydroxyacetone and glycerol caused a rapid fall in the hepatic concentrations of ketone bodies, dihydroxyacetone being more effective. 5. This decrease was not accompanied by significant changes in the concentrations of acetyl-CoA, long-chain acyl-CoA or free CoA. 6. The hepatic glycerophosphate concentration rose about 40-fold on administration of glycerol, whereas with dihydroxyacetone the increase was only about 50%. The large increase in glycerophosphate concentration after administration of glycerol was completely prevented by pretreatment of the rats with tri-iodothyronine. Tri-iodothyronine-treated rats showed the same decrease in ketone-body concentrations after administration of glycerol as the untreated rats. 7. Glycerol and dihydroxyacetone caused an increase in the hepatic lactate concentration; the pyruvate concentration rose only after injection of dihydroxyacetone. 8. Both compounds increased liver glycogen. 9. Calculation of the $[\text{free NAD}^+]/[\text{free NADH}]$ ratios indicated that dihydroxyacetone increased the ratio in cytoplasm and mitochondria, whereas glycerol caused a prompt fall in both compartments, followed at 10 min. by a slight rise in the mitochondrial compartment. 10. Dihydroxyacetone did not alter the hepatic content of ATP. 11. The findings suggest that the main reason for the antiketogenic effect of glycerol and dihydroxyacetone was a consequence of their ready metabolism and the provision of an increased supply of C_3 intermediates for conversion into oxaloacetate. Under the test conditions, neither the hepatic content of α -glycerophosphate nor the redox state of the NAD couples appeared to play a major role in the regulation of ketogenesis.

The mechanisms by which glucose, glycerol, lactate and related substances decrease the formation of ketone bodies by the liver *in vivo* are still uncertain. Factors that have been postulated to be involved in the control of hepatic ketogenesis

are the supply of free fatty acids to the liver (Fritz, 1961; Ontko & Zilversmit, 1966), the proportion of free fatty acids that undergoes oxidation as opposed to forming triglyceride (Fritz, 1961), the availability of alternative substrates of oxidation (Edson, 1936), and inhibition of the citrate synthase reaction either directly by long-chain acyl-CoA (Wieland, Weiss, Eger-Neufeldt, Teinzer & Westermann, 1966) or by ATP (Shepherd &

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Garland, 1966) or indirectly by a diminished supply of oxaloacetate. The diminished supply of oxaloacetate could be caused by a decrease of the mitochondrial $[NAD^+]/[NADH]$ ratio (Wieland, Weiss & Eger-Neufeldt, 1964) or by its diversion to glucose synthesis (Krebs, 1966).

The experiments reported in this paper were undertaken to obtain further information on the relative importance of the factors that may control ketone-body synthesis in the intact rat. Measurements were made of the changes in the concentrations of hepatic metabolites that occur when the rate of ketogenesis decreases in the liver on intramuscular administration of antiketogenic agents to starved rats. Glycerol and dihydroxyacetone were chosen as the antiketogenic substrates because both are readily metabolized and both share the stages of the gluconeogenic pathway between dihydroxyacetone phosphate and glucose (Bublitz & Kennedy, 1954); however, with glycerol there is an additional dehydrogenation step that may be expected to modify the redox state of mitochondria or cytoplasm and indicate to what extent the redox state may be of importance in the control of ketogenesis.

MATERIALS AND METHODS

Rats. Male rats of the Wistar strain weighing 140–180 g. were used. During the work it was found necessary to use rats from two different breeding colonies, which explains why the concentrations of certain metabolites in liver and blood of control rats differ in various experiments. However, within each experiment the control and experimental rats were from the same colony and were killed on the same day. The normal diet of the rats consisted of commercial rat cubes containing approx. 15% of protein, 3% of fat and 80% of carbohydrate (Oxoid breeding diet for rats and mice; Oxoid Ltd., London S.E.1). Unless otherwise stated all rats were starved for 48 hr. before use.

Reagents. Florisil (60–100 mesh) and dihydroxyacetone were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Oxoglutarate dehydrogenase was prepared from pigeon breast muscle by the method of Sanadi, Littlefield & Bock (1952). All other enzymes and coenzymes were obtained from Boehringer (London) Corp., London W.5.

Blood. Blood was collected in heparinized tubes after decapitation of the rats. A sample (0.3 ml.) was immediately deproteinized with 3 ml. of ice-cold 3% (w/v) $HClO_4$. The precipitated protein was removed by centrifugation and the supernatant fluid was neutralized with 10% (w/v) KOH. After the mixture had been left in the cold for at least 30 min., the crystalline precipitate of $KClO_4$ was removed and the supernatant fluid used for the determination of metabolites. Other samples of blood (0.2 ml.) were taken for the determination of free fatty acids.

Treatment of liver. The rats were killed by cervical dislocation. A portion of liver was rapidly removed (within 10 sec.) and clamped between metal tongs previously cooled in liquid N_2 (Wollenberger, Ristau & Schoffa, 1960). The frozen liver was then treated as described by Williamson,

Lund & Krebs (1967), except that before Florisil treatment a sample of the protein-free neutral supernatant was taken for the determination of acetyl-CoA and free CoA. The protein precipitate was kept for the determination of long-chain acyl-CoA.

Determination of metabolites. The following metabolites were determined by standard enzymic procedures: acetoacetate and 3-hydroxybutyrate (Williamson, Mellanby & Krebs, 1962); acetyl-CoA (Pearson, 1965); lactate, pyruvate, malate and α -glycerophosphate (Hohorst, Kreutz & Bücher, 1959); citrate (Gruber & Moellering, 1966); glutamate (Bernt & Bergmeyer, 1963); α -oxoglutarate (Bergmeyer & Bernt, 1963b); glucose (Bergmeyer & Bernt, 1963a); glycogen (Krebs, Dierks & Gascoyne, 1964). Long-chain acyl-CoA in the acid-insoluble fraction of liver was hydrolysed to liberate the CoA by the method of Tubbs & Garland (1964). Free CoA was determined by the procedure of Garland (1964) with acetylpyridine-adenine dinucleotide as coenzyme. Free fatty acid concentration of blood was measured by the colorimetric method of Itaya & Ui (1965). The spectrophotometric measurements were carried out on a Zeiss PMQ II or Eppendorf photometer.

Route of administration of antiketogenic agents. The intramuscular route of injection was chosen because it resulted in slow absorption and a fairly constant concentration of glycerol or dihydroxyacetone in the blood over a period of at least 1 hr. The oral route was not used, so as to avoid the possible release of jejunal glucagon-like activity (Unger, Ohneda, Valverde, Eisentraut & Exton, 1968) and to minimize the secretion of insulin, because it is well established that oral glucose results in higher plasma insulin concentrations than intravenous injection.

RESULTS

Concentrations of metabolites in blood after administration of glucose, glycerol or dihydroxyacetone to starved rats

An intramuscular injection of glucose or its precursors into starved rats decreased the concentrations of ketone bodies in the blood within 15 min. (Table 1). At 60 min. after the injection the relative decreases in blood ketone bodies were 70% for glucose, 71% for glycerol and 83% for dihydroxyacetone. As reported by other workers (Farkas, Vertura, Usardi & Paoletti, 1964; Hales & Kennedy, 1964) the concentration of free fatty acids in blood was elevated about threefold in the starved rat. There was no major decrease (less than 25%) in the concentration of free fatty acids after administration of any of the three compounds (Table 1). Although there was little change in the blood glucose concentration for at least 30 min. after the administration of dihydroxyacetone, there was an immediate increase (twofold; $P=0.005$) in the blood lactate and pyruvate concentrations that was maintained for 60 min. The failure of glucose to produce a similar sustained increase suggests that the source of lactate in the dihydroxyacetone-treated rats might be the liver rather than the

Table 1. *Effects of administration of glucose, glycerol or dihydroxyacetone on the concentrations of metabolites in blood of starved rats*

Nutritional state	Substance injected	Time killed after injection (min.)	Treatment of rats						[Total ketone bodies]
			[Free fatty acids]	[Glucose]	[Lactate]	[Pyruvate]	[Hydroxybutyrate]	[Acetoacetate]	
Fed	None	—	0.15 ± 0.04 (8)	6.02 ± 0.44 (6)	2.52 ± 0.62 (5)	0.22 ± 0.09 (4)	0.072 ± 0.042 (5)	0.064 ± 0.014 (7)	0.136
	NaCl	30	0.50 ± 0.10 (20)	4.27 ± 0.51 (12)	2.23 ± 0.81 (12)	0.16 ± 0.06 (12)	1.62 ± 0.47 (19)	0.47 ± 0.13 (19)	2.09
Starved	Glucose	15	0.38 ± 0.03 (5)	8.73 ± 0.90 (5)	3.86 ± 1.10 (5)	—	0.32 ± 0.13 (5)	0.40 ± 0.13 (5)	0.72
	Glucose	30	0.48 ± 0.10 (5)	5.92 ± 0.55 (5)	2.02 ± 0.63 (5)	—	0.35 ± 0.11 (5)	0.40 ± 0.18 (5)	0.75
	Glucose	60	0.47 ± 0.19 (4)	6.06 ± 0.32 (4)	2.19 ± 0.49 (4)	—	0.30 ± 0.10 (4)	0.33 ± 0.18 (4)	0.63
	Glycerol	30	0.42 ± 0.04 (3)	5.20 ± 0.75 (3)	3.14 ± 0.22 (3)	0.17 ± 0.04 (3)	0.64 ± 0.33 (3)	0.29 ± 0.11 (4)	0.93
	Glycerol	60	0.38 ± 0.15 (6)	5.26 (2)	1.89 (2)	0.13 (2)	0.41 ± 0.16 (6)	0.19 ± 0.14 (6)	0.80
	Dihydroxyacetone	15	0.37 ± 0.17 (4)	4.04 ± 0.88 (4)	4.29 ± 0.87 (4)	0.26 ± 0.14 (4)	0.43 ± 0.09 (4)	0.13 ± 0.05 (4)	0.56
	Dihydroxyacetone	30	0.40 ± 0.14 (8)	4.60 ± 1.09 (8)	5.37 ± 1.54 (8)	0.33 ± 0.12 (8)	0.36 ± 0.15 (8)	0.17 ± 0.07 (8)	0.53
	Dihydroxyacetone	60	0.34 ± 0.22 (8)	6.19 ± 1.44 (4)	5.70 ± 2.31 (4)	0.29 ± 0.09 (4)	0.25 ± 0.12 (8)	0.10 ± 0.04 (8)	0.35

peripheral tissues, because the liver is the main site of dihydroxyacetone utilization (Borondy & Tsao, 1967).

Concentrations of metabolites in liver after the administration of glycerol or dihydroxyacetone to starved rats

Ketone bodies. Parallel with the fall in the concentrations of ketone bodies in blood there was a rapid decrease in the hepatic ketone-body concentration on administration of glycerol or dihydroxyacetone (Fig. 1). The time-course of the changes was biphasic; in the first 10 min. the concentration of ketone bodies fell by about 40%, and thereafter the decrease was more gradual and the concentration normally present in livers from fed rats had not been reached by 60 min. Decreases in both acetoacetate and 3-hydroxybutyrate contributed to the fall in total ketone-body concentration (Table 5). Dihydroxyacetone was more effective than glycerol throughout the period studied.

Free CoA, acetyl-CoA and long-chain acyl-CoA. The absence of an appreciable change in the blood concentration of free fatty acids (the major precursors of ketone bodies) during this period of decreased ketogenesis suggested that factors other than the concentration of the free fatty acids can control their rate of degradation. The concentrations of free CoA, acetyl-CoA and long-chain acyl-CoA were therefore measured at 1 and 2 hr. after administration of glycerol or dihydroxyacetone and compared with those for fed and starved rats (Table 2). There was an approximately three-fold rise in acetyl-CoA and long-chain acyl-CoA concentrations on starvation, and the free CoA concentration was slightly elevated as reported by Bortz & Lynen (1963) and Tubbs & Garland (1964). Glycerol or dihydroxyacetone caused only a slight

Fig. 1. Decrease in hepatic ketone-body concentrations after administration of glycerol or dihydroxyacetone to starved rats. The values are calculated from the data in Table 5. Each point represents the mean value for five rats. ○, Glycerol-treated rats; ●, dihydroxyacetone-treated rats.

Table 2. *Hepatic concentrations of acetyl-CoA, long-chain acyl-CoA and free CoA in fed and starved rats and starved rats injected with glycerol or dihydroxyacetone*

Administration of glycerol and dihydroxyacetone was as described in Table 1. For other experimental details see the Materials and Methods section. The concentrations of the metabolites are mean values $\pm$ s.d. expressed as nmoles/g. fresh wt. of liver. The numbers of rats are given in parentheses; starvation was for 48 hr.

Treatment of rats		Time killed after injection (min.)	[Acetyl-CoA]	[Long-chain acyl-CoA]	[Free CoA]
Nutritional state	Substance injected				
Fed	None	—	27 $\pm$ 8 (7)	21 $\pm$ 6 (7)	125 $\pm$ 15 (7)
Starved	None	—	76 $\pm$ 19 (21)	71 $\pm$ 24 (17)	158 $\pm$ 39 (19)
	Glycerol	60	78 $\pm$ 19 (8)	58 $\pm$ 30 (10)	181 $\pm$ 14 (8)
	Glycerol	120	63 $\pm$ 14 (8)	57 $\pm$ 24 (10)	189 $\pm$ 14 (8)
	Dihydroxyacetone	60	79 $\pm$ 20 (6)	57 $\pm$ 18 (6)	193 $\pm$ 19 (4)
	Dihydroxyacetone	120	66 $\pm$ 12 (6)	47 $\pm$ 20 (6)	184 $\pm$ 20 (4)

Table 3. *Hepatic concentrations of α -glycerophosphate in starved rats and in starved rats injected with glycerol or dihydroxyacetone*

The administration of glycerol and dihydroxyacetone was as described in Table 1. For other experimental details see the Materials and Methods section. The concentrations of α -glycerophosphate are mean values $\pm$ s.d. expressed as μ moles/g. fresh wt. of liver. The numbers of rats are given in parentheses. The value for the control rats (starved for 48 hr.) is that shown at 0 min.

Time after injection (min.)	[Glycerophosphate]	
	Glycerol-treated	Dihydroxyacetone- treated
0	0.26 $\pm$ 0.07 (10)	0.26 $\pm$ 0.07 (10)
5	3.9 $\pm$ 1.3 (4)	0.38 (2)
10	5.8 $\pm$ 1.7 (5)	0.39 $\pm$ 0.11 (3)
20	7.8 $\pm$ 1.3 (3)	0.28 (2)
40	9.8 $\pm$ 2.3 (5)	0.44 $\pm$ 0.15 (4)
60	10.1 $\pm$ 3.0 (4)	0.37 $\pm$ 0.06 (3)

decrease (less than 25%) in the acetyl-CoA and long-chain acyl-CoA concentrations and no significant change in the free CoA concentration at a time (1 hr.) when there was a large fall in ketone-body concentration.

α -Glycerophosphate. α -Glycerophosphate has been implicated in the control of ketogenesis because its availability could divert free fatty acids from the β -oxidation pathway to conversion into triglycerides (Fritz, 1961). Administration of dihydroxyacetone caused a relatively slight increase (about 50%) in the concentration of α -glycerophosphate that was maintained for 60 min. (Table 3). There was a 15-fold rise in glycerophosphate 5 min. after the injection of glycerol, and at 40 min. it was about 40-fold higher than that of the control rats. A high glycerophosphate concentration (7–9 μ moles/g.) has also been observed on perfusion of isolated rat liver with glycerol (Wieland & Matschinsky, 1962). Assuming that there was no

utilization of α -glycerophosphate, the activity of glycerokinase *in vivo* during the first 5 min. after injection of glycerol was 0.7 μ mole of glycerol phosphorylated/min./g. of liver. This value is a minimum one and compares with a value of 1.7 μ moles/min./g. of liver at 26° measured *in vitro* under optimum conditions (Robinson & Newsholme, 1969).

The accumulation of glycerophosphate in liver after administration of glycerol suggested that its oxidation (via cytoplasmic NAD-linked glycerophosphate dehydrogenase and the intramitochondrial flavin-linked glycerophosphate oxidase) was rate-limiting. This suggestion was tested by treating rats with tri-iodothyronine, which is known to increase considerably the activity of the hepatic mitochondrial enzyme (up to 20-fold) while not altering the activity of the cytoplasmic enzyme (Lee & Lardy, 1965), and measuring the metabolite patterns after glycerol administration. Treatment

Table 4. *Effects of pre-injection of tri-iodothyronine on the hepatic concentrations of glycerophosphate and other metabolites after administration of glycerol*

A group of rats was injected intraperitoneally with 200 μ g. of tri-iodothyronine/100 g. body wt. and immediately starved for 48 hr. The rats were then given an intramuscular injection of 1.0 ml. of 40% (w/v) glycerol. A second group of rats was starved for 48 hr. and then injected with glycerol. Rats from these groups were killed at the times indicated. A third group of rats was starved for 48 hr. and killed without further treatment. For full details see the Materials and Methods section. The results are mean values $\pm$ s.d. and are expressed in μ moles/g. wet wt. of liver. The numbers of rats are given in parentheses.

Treatment of rats		Time killed after injection (min.)		[Glycero-phosphate]	[Lactate]	[Pyruvate]	[3-Hydroxy-butyrate]	[Aceto-acetate]	[3-Hydroxy-butyrate]
Substance injected									
Controls	None (10)	—		0.21 $\pm$ 0.12	0.35 $\pm$ 0.18	0.017 $\pm$ 0.012	1.55 $\pm$ 0.60	0.52 $\pm$ 0.15	3.0
	Glycerol (10)	60		5.69 $\pm$ 3.33	0.98 $\pm$ 0.16	0.031 $\pm$ 0.020	0.84 $\pm$ 0.19	0.29 $\pm$ 0.09	2.9
Tri-iodothyronine-treated	Glycerol (4)	30		0.72 $\pm$ 0.18	1.09 $\pm$ 0.20	0.045 $\pm$ 0.014	1.00 $\pm$ 0.48	0.28 $\pm$ 0.16	3.5
	Glycerol (6)	60		0.53 $\pm$ 0.22	0.90 $\pm$ 0.32	0.042 $\pm$ 0.014	0.81 $\pm$ 0.41	0.28 $\pm$ 0.14	2.9

Table 5. *Effects of glycerol and dihydroxyacetone on the concentrations of the substrates of NAD-linked dehydrogenase systems in livers of starved rats*

Rats starved for 44-48 hr. were given an intramuscular injection of 1.0 ml. of (a) 0.9% NaCl, (b) 40% (w/v) dihydroxyacetone or (c) 40% (w/v) glycerol. The rats were killed at timed intervals after injection. For other experimental details see the Materials and Methods section. The concentrations of metabolites are mean values $\pm$ s.d. expressed as μ moles/g. fresh wt. of liver. The numbers of rats are given in parentheses. For convenience the control rats have been treated as one group.

Treatment of animals		Time after injection (min.)		[Lactate]	[Pyruvate]	[Glutamate]	[Oxoglutarate]	[Hydroxy-butyrate]	[Acetoacetate]
NaCl (19)		5-60		0.54 $\pm$ 0.27	0.021 $\pm$ 0.008	2.38 $\pm$ 0.65	0.108 $\pm$ 0.045	2.64 $\pm$ 0.79	0.82 $\pm$ 0.19
Dihydroxyacetone (5)		5		1.33 $\pm$ 0.37	0.088 $\pm$ 0.018	2.19 $\pm$ 0.22	0.110 $\pm$ 0.024	1.68 $\pm$ 0.47	0.66 $\pm$ 0.22
	(5)	10		2.20 $\pm$ 0.67	0.123 $\pm$ 0.037	2.64 $\pm$ 0.15	0.138 $\pm$ 0.039	1.41 $\pm$ 0.29	0.47 $\pm$ 0.13
	(5)	20		2.32 $\pm$ 0.29	0.231 $\pm$ 0.071	2.96 $\pm$ 0.57	0.238 $\pm$ 0.027	0.71 $\pm$ 0.48	0.42 $\pm$ 0.25
	(5)	40		2.43 $\pm$ 1.42	0.192 $\pm$ 0.080	3.75 $\pm$ 0.95	0.233 $\pm$ 0.035	0.73 $\pm$ 0.40	0.34 $\pm$ 0.10
	(6)	60		2.81 $\pm$ 1.05	0.311 $\pm$ 0.110	2.93 $\pm$ 0.48	0.253 $\pm$ 0.060	0.41 $\pm$ 0.19	0.22 $\pm$ 0.04
Glycerol (5)		5		1.37 $\pm$ 0.89	0.032 $\pm$ 0.021	1.89 $\pm$ 0.46	0.046 $\pm$ 0.015	2.05 $\pm$ 0.71	0.61 $\pm$ 0.20
	(6)	10		1.19 $\pm$ 0.42	0.038 $\pm$ 0.018	2.38 $\pm$ 0.39	0.067 $\pm$ 0.019	1.82 $\pm$ 0.28	0.44 $\pm$ 0.11
	(5)	20		1.03 $\pm$ 0.17	0.027 $\pm$ 0.012	3.38 $\pm$ 0.68	0.106 $\pm$ 0.016	1.35 $\pm$ 0.53	0.53 $\pm$ 0.19
	(5)	40		1.13 $\pm$ 0.32	0.032 $\pm$ 0.017	2.98 $\pm$ 0.59	0.099 $\pm$ 0.031	1.26 $\pm$ 0.59	0.50 $\pm$ 0.23
	(5)	60		0.98 $\pm$ 0.16	0.031 $\pm$ 0.020	3.45 $\pm$ 0.42	0.165 $\pm$ 0.086	0.76 $\pm$ 0.26	0.27 $\pm$ 0.14

with tri-iodothyronine caused a tenfold decrease in the concentration of hepatic α -glycerophosphate after glycerol administration (Table 4). The decreases in total ketone bodies after injection of glycerol (60 min.) were virtually identical in the control and the tri-iodothyronine-treated rats (Table 4). Although there was no significant difference between the hepatic lactate concentrations of control and tri-iodothyronine-treated rats receiving glycerol, there was a higher pyruvate concentration in the latter group, resulting in a more oxidized lactate/pyruvate ratio (Table 4).

Substrates of lactate dehydrogenase and glutamate dehydrogenase. On administration of dihydroxyacetone the pyruvate concentration increased fourfold within 5 min. and continued to rise throughout the experiment (Table 5). In contrast, injection of glycerol caused no major changes in the pyruvate concentration (Table 5). There was a rise in lactate concentration with both compounds; as in the blood, the increase was considerably greater with dihydroxyacetone (Table 5). Thus with both substrates there was an increased flux of carbon through the terminal stages of glycolysis. The concentrations of α -oxoglutarate rose twofold with dihydroxyacetone, whereas glycerol caused a transient fall (Table 5). There were no marked changes in the glutamate concentration after injection of glycerol or dihydroxyacetone.

Cytoplasmic and mitochondrial [free NAD⁺]/[free NADH] ratios. The cytoplasmic [free NAD⁺]/[free NADH] ratio calculated from the [lactate]/[pyruvate] ratio (Williamson *et al.* 1967) rose immediately on injection of dihydroxyacetone, and this rise continued until a value exceeding that found in livers from fed rats was reached (Fig. 2).

Fig. 2. Changes in the cytoplasmic [free NAD⁺]/[free NADH] ratio after administration of glycerol or dihydroxyacetone. The values were calculated from the data in Table 5 and each point represents the mean value for five animals. ○, Glycerol-treated rats; ●, dihydroxyacetone-treated rats; ---, control rats.

On the other hand, with glycerol the cytoplasmic ratio fell below that of the control rats and remained low for the whole period (Fig. 2). This fall is no doubt connected with the supply of NADH via the cytoplasmic α -glycerophosphate dehydrogenase. The mitochondrial [free NAD⁺]/[free NADH] ratio, as calculated from the [acetoacetate]/[hydroxybutyrate] ratio, initially decreased on administration of glycerol, but then rose to values approaching that for livers from fed rats (Fig. 3). With dihydroxyacetone there was a rapid increase in the mitochondrial ratio, and it reached values higher than those obtained after administration of glycerol (Fig. 3). If a constant hepatic ammonia concentration of 0.6 μ mole/g. fresh wt. of liver (Williamson *et al.* 1967) is assumed, calculation of the mitochondrial [free NAD⁺]/[free NADH] ratio from the [glutamate]/[oxoglutarate][NH₄⁺] ratio (Williamson *et al.* 1967) gives values similar to those shown in Fig. 3, except that the difference between glycerol and dihydroxyacetone is more marked.

Malate and citrate. Administration of dihydroxyacetone caused an approximate doubling of the hepatic concentration of citrate; there was no marked change after injection of glycerol (Table 6). Neither substrate caused appreciable changes in the malate concentration (Table 6).

Glucose and glycogen. Measurements of the hepatic glucose and glycogen concentrations after injection of glycerol or dihydroxyacetone (Table 7) showed that, as in the perfused rat liver (Ross, Hems & Krebs, 1967), there is an increased flux of triose phosphate carbon atoms not only to lactate (above) but also towards carbohydrate. The

Fig. 3. Changes in the mitochondrial [free NAD⁺]/[free NADH] ratio after administration of glycerol or dihydroxyacetone. The values were calculated from data in Table 5. Each point represents the mean value for five animals. ○, Glycerol-treated rats; ●, dihydroxyacetone-treated rats; ---, control rats.

Table 6. *Hepatic concentrations of citrate and malate in starved rats and starved rats injected with glycerol or dihydroxyacetone*

Administration of glycerol and dihydroxyacetone was as described in Table 1. For other experimental details see the Materials and Methods section. The concentrations of metabolites are mean values $\pm$ s.d. expressed as μ moles/g. fresh wt. of liver. The numbers of rats are given in parentheses. The values for control rats are given at 0 min.

Time killed after injection (min.)	Glycerol-treated		Dihydroxyacetone-treated	
	[Citrate]	[Malate]	[Citrate]	[Malate]
0	0.49 $\pm$ 0.12 (22)	0.49 $\pm$ 0.15 (16)	0.49 $\pm$ 0.12 (22)	0.49 $\pm$ 0.15 (16)
5	0.48 $\pm$ 0.19 (5)	0.65 $\pm$ 0.17 (9)	1.10 $\pm$ 0.71 (4)	0.44 $\pm$ 0.15 (5)
10	0.59 $\pm$ 0.17 (5)	0.52 $\pm$ 0.13 (3)	0.87 $\pm$ 0.22 (4)	0.42 $\pm$ 0.06 (4)
20	0.52 $\pm$ 0.17 (6)	0.50 $\pm$ 0.07 (3)	0.75 $\pm$ 0.27 (3)	0.36 $\pm$ 0.07 (3)
40	0.51 $\pm$ 0.15 (6)	—	1.22 $\pm$ 0.68 (5)	0.48 (2)
60	0.64 $\pm$ 0.17 (6)	0.51 $\pm$ 0.12 (3)	1.01 $\pm$ 0.56 (7)	0.50 (2)

Table 7. *Concentrations of glucose and glycogen in livers of starved rats treated with glycerol or dihydroxyacetone*

The administration of glycerol and dihydroxyacetone was as described in Table 1. The values for glucose and glycogen (expressed as glucose) are mean values of two or three experiments and are expressed as μ moles/g. fresh wt. of liver. The values for control rats are given at 0 min.

Time after injection (min.)	Glycerol-treated		Dihydroxyacetone-treated	
	[Glucose]	[Glycogen]	[Glucose]	[Glycogen]
0	6.3	5.0	6.3	5.0
5	10.6	5.1	8.4	6.3
10	8.3	12.2	8.1	3.1
20	12.1	8.5	12.3	15.0
40	—	—	13.2	32.4
60	19.3	55.9	20.3	58.3

Table 8. *Concentrations of ATP, ADP and AMP in livers of starved rats after injection of dihydroxyacetone*

Starved rats (48 hr.) were given an intramuscular injection of (a) 1.0 ml. of 0.9% NaCl or (b) 1.0 ml. of 40% (w/v) dihydroxyacetone, and then killed 10 min. later. For other experimental details see the Materials and Methods section. The results are mean values $\pm$ s.d. expressed in μ moles/g. fresh wt. of liver. The numbers of rats are given in parentheses.

Substance injected	[ATP]	[ADP]	[AMP]	[Ketone bodies]
NaCl (4)	2.31 $\pm$ 0.19	1.45 $\pm$ 0.11	0.33 $\pm$ 0.12	1.77 $\pm$ 0.40
Dihydroxyacetone (5)	2.28 $\pm$ 0.22	1.44 $\pm$ 0.16	0.32 $\pm$ 0.055	0.54 $\pm$ 0.17

glycogen content (measured as glucose) increased by approx. 50 μ moles/g. fresh wt. of liver in 60 min. This rate of glycogen deposition is similar to that reported to occur after administration of glycerol (Gidez & Karnovsky, 1954) or lactate (Hornbrook, Burch & Lowry, 1965) to starved rats.

Adenine nucleotides. The possibility that phosphorylation of dihydroxyacetone or glycerol might lead to a depletion of hepatic ATP and a consequent deinhibition of citrate synthase (Garland, 1968) was tested. There was no significant change in the

hepatic concentration of ATP, ADP or AMP after injection of dihydroxyacetone at a time (10 min.) when the concentration of ketone bodies had fallen to about one-third of the original value (Table 8).

DISCUSSION

Role of concentration of free fatty acids in ketogenesis. The approximate parallel between the concentration of free fatty acids and the concentrations of ketone bodies in blood or liver (Ontko

& Zilversmit, 1966; Williamson, Wright, Malaisse & Ashmore, 1966; Start & Newsholme, 1968) indicates that the concentration of circulating free fatty acids is one of the important factors in the regulation of hepatic ketogenesis. The finding (Tables 1 and 2) that the hepatic concentrations of ketone bodies decrease on administration of glycerol or dihydroxyacetone without a marked fall in the blood concentration of free fatty acids demonstrates that it is not the absolute concentration of free fatty acids in the plasma that determines the rate of ketogenesis. This depends also on the availability of other readily metabolizable substrates.

Role of α -glycerophosphate. Fritz (1961) suggested that the availability of α -glycerophosphate is a factor in deciding the intrahepatic fate of free fatty acids because it may determine their rate of esterification. There is evidence that the hepatic concentration of glycerophosphate decreases in starvation, although the values reported in the literature vary considerably (Bortz & Lynen, 1963; Tzur, Tal & Shapiro, 1964; Zakim, 1965). In the present experiments the administration of glycerol increased the concentration of glycerophosphate more than did dihydroxyacetone (Table 3), but the former was less effective in inhibiting ketogenesis (Fig. 1). Further, prevention of the accumulation of glycerophosphate by pretreatment of rats with tri-iodothyronine did not affect the antiketogenic action of glycerol (Table 4). Thus the availability of α -glycerophosphate did not appear to be a major factor controlling the rate of ketogenesis under

these conditions. This does not exclude the possibility that α -glycerophosphate may be a rate-limiting factor in some situations.

Role of CoA and CoA esters in ketogenesis. The increased concentration of acetyl-CoA in livers from starved, fat-fed and alloxan-diabetic rats has led to the suggestion that the concentration of acetyl-CoA in the liver is directly related to the rate of ketogenesis (Wieland & Weiss, 1963; Williamson, Herzeg, Coles & Danish, 1966). Many workers have suggested that the [acetyl-CoA]/[CoA] ratio is the decisive factor because it may determine the steady-state concentration of acetoacetyl-CoA via the equilibrium of the thiolase system (see Greville & Tubbs, 1968). The equilibrium constant of thiolase is 6×10^{-5} (Goldman, 1954) so that:

$$[\text{Acetoacetyl-CoA}] = 6 \times 10^{-5} \times \frac{[\text{Acetyl-CoA}]^2}{[\text{CoA}]}$$

In the present experiments (Table 2) there was no significant decrease in the concentration of acetyl-CoA or CoA after the administration of glycerol or dihydroxyacetone (see also Foster, 1967), and hence the acetoacetyl-CoA concentrations calculated on the basis of the above formula did not alter for at least 1 hr. The calculated acetoacetyl-CoA concentration in livers from starved rats (2.2 pmoles) was sixfold higher than that of fed rats (0.35 pmoles). Information on the intracellular distribution of acetyl-CoA is not available, but if, as seems likely, a considerable portion of the

Table 9. *Calculation of oxaloacetate concentration in cytoplasm and mitochondria*

The values have been calculated from the formula:

$$[\text{Oxaloacetate}] = \frac{[\text{Malate}] \times [\text{NAD}^+]}{[\text{NADH}] \times K}$$

where K is the equilibrium constant of the malate dehydrogenase system (2.78×10^{-5} at pH 7.0; Williamson *et al.* 1967), and $[\text{NAD}^+]/[\text{NADH}]$ refers to the free nicotinamide-adenine nucleotides in the cytoplasm and mitochondria. For the purpose of these calculations it has been assumed that the measured malate concentration in $\mu\text{moles/g. fresh wt.}$ is the same in cytoplasm and mitochondria. Other assumptions on which the calculations are based have been dealt with more fully by Williamson *et al.* (1967). The data on malate concentration are in Table 6, and the cytoplasmic and mitochondrial [free NAD^+]/[free NADH] ratios in Figs. 2 and 3.

Time after injection (min.)	[Oxaloacetate] (nmoles/g.)			
	Glycerol-treated		Dihydroxyacetone-treated	
	Cytoplasm	Mitochondria	Cytoplasm	Mitochondria
0	4.8	0.09	4.8	0.09
5	3.8	0.11	7.3	0.10
10	4.2	0.07	5.9	0.08
20	3.3	0.11	9.0	0.12
40	—	—	9.5	0.13
60	4.0	0.10	13.9	0.15

measured acetyl-CoA content is cytoplasmic any changes in the mitochondrial compartment might not be detected. The postulated high concentration of acetyl-CoA in the cytoplasm and the biphasic nature of the decrease in hepatic ketone bodies (Fig. 1) lend support to the role in starvation of the extramitochondrial pathway of acetoacetate synthesis (Williamson, Bates & Krebs, 1968).

The failure to find a change in the long-chain acyl-CoA concentration (Table 2) confirms the idea that the inhibition of citrate synthase by acyl-CoA is of little physiological importance *in vivo* (Srere, 1968).

Intramitochondrial concentration of oxaloacetate. The intramitochondrial concentration of oxaloacetate is taken to be one of the main factors controlling the fate of acetyl-CoA (Wieland & Löffler, 1963; Löffler, Matschinsky & Wieland, 1965; Krebs, 1966). Calculation of the oxaloacetate concentrations, based on the assumption that the mitochondrial malate dehydrogenase system is in equilibrium with its reactants and that the extramitochondrial and intramitochondrial concentrations of malate are the same, indicate no major changes in the oxaloacetate concentration after administration of glycerol or dihydroxyacetone in mitochondria during the period of greatest fall in ketone-body concentration (Table 9). Thus on this basis, which has the hypothetical element of assuming the existence of certain equilibria, the concentration of oxaloacetate does not appear to be a major factor responsible for the antiketogenic effect of glycerol or dihydroxyacetone under the experimental conditions. However, with both compounds there was an increase in the lactate-plus-pyruvate pool and this may well result in a higher intramitochondrial oxaloacetate concentration via pyruvate carboxylation.

General conclusions. This work shows that injection of glycerol or dihydroxyacetone promptly decreases hepatic ketogenesis in starved rats. This is brought about without any major change in the concentration of plasma free fatty acids or hepatic concentrations of acetyl-CoA, long-chain acyl-CoA, free CoA or ATP, nor are alterations in the hepatic concentration of α -glycerophosphate correlated with the antiketogenic effects. As the redox states of the NAD couple in the cytoplasm and mitochondria are initially altered in opposite directions by glycerol and dihydroxyacetone (whereas the inhibitory effects on ketogenesis are similar) the redox state cannot be a major factor regulating the rate of ketogenesis in the starved rat. The increased supply of precursors for the formation of oxaloacetate may be the most important factor contributing to the decrease in ketogenesis. It must be emphasized that this conclusion applies only to the specific situation of these experiments.

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